

## Androgen disruption of early development in Qurt strain medaka (*Oryzias latipes*)

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### Abstract

Androgen disrupting chemicals (ADCs) are widespread in the aquatic environment, where they may pose a risk to aquatic organisms during critical periods of development. In this study, static renewal 96-h toxicity tests were followed over a 3-month growth period to investigate the endocrine disrupting effects of the androgen 11-ketotestosterone (11-KT), and the antiandrogen flutamide (FLU) on 1-week-old Qurt strain medaka larvae. The measured endpoints included: survival, growth performance (i.e., body weight, body length, condition index), and histopathology. There was no significant acute mortality, except for males treated with the highest FLU concentration (96 h-LC<sub>50</sub> = 1.92 mg/l). Gender-specific effects in growth were identified after 11-KT and FLU treatments. Histopathological alterations including thyroid follicular hypertrophy (TFH), germ cell necrosis (GCN), ovarian atresia (OA), and testis-ova (TO) were observed in medaka at 90-day post-exposure. We observed TFH in all 11-KT treatment levels. The incidence of TFH in males was double that in females 11-KT or FLU treatment. Females showed GCN at lower 11-KT concentrations (0.01, 0.1 mg/l) than males (1.0 mg/l). Severe OA was observed at low (0.01 mg/l) and high (1.0 mg/l) 11-KT concentrations in females. Flutamide induced TO (0.32, 1.0 mg/l), ovarian cell necrosis (0.32 mg/l), and disrupted spermatogenesis (3.2 mg/l) in males. The lowest observed effective concentration (LOEC) for TO induction in Qurt medaka males was 0.32 mg/l. The present study underscores the importance of fish early life stage tests for detecting the interaction of ADCs with the reproductive and thyroid glands in both genders.

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### 1. Introduction

Androgenic and antiandrogenic environmental contaminants can affect endocrine function by altering the androgen signaling pathways. In teleosts, androgens such as 11-ketotestosterone (11-KT), control sexual development, reproduction, and the development and maintenance of male secondary sex characteristics (Borg, 1994). A suite of environmental chemicals have been shown to alter androgen-mediated sexual development resulting in disruption of gonadal sex differentiation and gametogenesis (Kelce and Wilson, 1997). The environmental androgen disrupting chemicals (ADCs) identified to date include pesticides and industrial contaminants released in effluents from anthropogenic sources (Gray et al., 2006). For example, pulp

and paper mill effluents (Howell et al., 1980; Parks et al., 2001; Larsson and Förlin, 2002) and tributyltin (Shimasaki et al., 2003) are known to cause masculinization of female fish. Concentrated animal feeding operations that generate large quantities of androgens from urinary and fecal deposition of pollutants in wastewater have also been linked to androgenic effects in the rat (Gray et al., 2006) and fish (fathead minnow; *Pimephales promelas*) (Orlando et al., 2004). Androgenic activity, measured by yeast-based in vitro assays, has been detected in treated wastewater in the United Kingdom (Kirk et al., 2002). Moreover, potent androgens such as testosterone and androstenedione, have been found in treated sewage effluent in the U.S. (Jenkins et al., 2001; Kolodziej et al., 2003). Although androgens may be present in treated effluents at higher concentrations than estrogens (Sumpter, 2005), ADCs have received far less attention.

Androgen disrupting effects such as precocious male secondary sexual characteristics and gonadal atrophy have been studied in Japanese medaka (*Oryzias latipes*) since the 1950s

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(Yamamoto, 1958; Hishida and Kawamoto, 1970). The medaka is an ideal model for studying ADCs due to its short generation time (6–8 weeks) and daily year-round spawning under controlled photoperiod and temperature conditions (Patyna et al., 1999; Parrott et al., 2001; Hutchinson et al., 2003). Gonad histopathology in medaka can be used as a core endpoint for the rapid detection of androgen disrupting activity (Parrott et al., 2001; Hutchinson et al., 2003). The medaka is a gonochoristic species with an XX/XY sex determination system. In Qurt strain medaka, the genotypic sex can be identified at early embryonic stages due to sex-linked occurrence of leucophores. Leucophore differentiation occurs at the 2-day-old embryo stage in males, but not in females (Wada et al., 1998). Therefore, it is possible to identify the genotypic sex before sexual differentiation, which facilitates the examination of gender-specific effects of contaminants during critical stages of development.

The purpose of our study was to characterize the effects of exogenously administered 11-KT and the antiandrogen flutamide (FLU) on the early development of Qurt medaka. We hypothesized that short-term androgen and antiandrogen exposure would have long term effects on Qurt strain medaka survival, growth, and tissue structure. We evaluated the long term effects of a short-term exposure of Qurt medaka to 11-KT and FLU by examining survival, growth, sexual differentiation, and histopathology.

## 2. Materials and methods

### 2.1. Chemicals and experimental conditions

The chemicals 11-KT (98% purity) and FLU (99% purity) were obtained from Sigma–Aldrich Corp. (St. Louis, MO, USA). Each chemical was administered via aqueous exposure with 0.1 ml/l of ethanol used as a solvent. Stock solutions of 11-KT and FLU, with nominal concentrations of 10 and 3.2 mg/l, were prepared in reconstituted water. Reconstituted water was prepared according to the guidelines of the United States Environmental Protection Agency (USEPA) (Horning and Weber, 1985). Target 11-KT and FLU concentrations in the static renewal system were generated by combining the stock solution with reconstituted water in the test vessels. Test vessels consisted of 600 ml Pyrex® beakers containing 500 ml of test solution that were pretreated overnight with the corresponding test solutions prior to use in exposures. Beakers were maintained at a temperature of  $25.0 \pm 1^\circ\text{C}$  in a recirculating water bath. Exposure concentrations were renewed by exchanging 70% of the test solution every 24-h during the 96-h test.

### 2.2. Medaka and chemical treatments

Embryos were separated by gender within 2 days postfertilization and prior to hatching based on sex-linked coloration (Wada et al., 1998). Male embryos were distinguished by the presence of leucophores in the head region and the anterior portion of the trunk. Female embryos were identified by the lack of leucophores in the head region. After hatching (usually 8–10 days post-fertilization), larvae were transferred to a new recep-

tacle and fed twice daily with a specially formulated purified casein diet (DeKoven et al., 1992) until they reached 7 days post-hatch and exposure began. During the 7-day post-hatch period, daily observations were performed to remove larvae that were not feeding and swimming actively, or showed signs of impaired growth. Seven-day-old larvae were selected for initiation of exposure since the critical period in medaka during which developmental processes are more susceptible to chemical insult has been identified as 6–10 days post-hatch (Arcand-Hoy and Benson, 1998).

Exposures to 11-KT and FLU were conducted using guidelines similar to those of the 96-h standard static renewal method for acute toxicity testing (USEPA, 2002) and test methods described in detail by Teh et al. (2003) and Environment Canada (1998). Prior to initiation of chemical exposures, fish were allowed to acclimate to test conditions by placement in an aerated beaker within an environmental chamber. The fish were maintained at  $25 \pm 1^\circ\text{C}$  under a 16:8 L:D photoperiod. Each beaker containing 50 male or 50 female 1-week-old larvae was randomly assigned to different positions within the environmental chamber. Medaka larvae were not fed during the 96-h exposure period in order to prevent interaction of the test chemicals with dietary particulate matter. There were two replicate beakers per gender at each 11-KT and FLU concentration, plus clean-water and organic solvent control beakers. Exposure concentrations for the 11-KT 96-h acute toxicity tests were 0, 0.01, 0.1, and 1.0 mg/l. Target concentrations of FLU (0.032, 0.1, 0.32, 1.0, and 3.2 mg/l) were tested in an initial range-finding study. A second range-finding study was conducted at target FLU concentrations of 0, 0.032, 0.32, and 3.2 mg/l. During the exposures, daily observations were recorded to examine mortality.

### 2.3. Sublethal toxicity testing

#### 2.3.1. Survival and growth

After the 96-h exposure to 11-KT or FLU, all surviving fish were transferred to 38 l test tanks in a flow-through system and grown for 3 months to approximate sexual maturity. During this period, fish were monitored daily to evaluate survival and sublethal effects until the fish reached sexual maturity. After 90 days, fish were euthanized with a lethal concentration (100 mg/l) of tricaine methanesulfonate (MS-222), and their body cavities were surgically exposed to ensure optimal fixation. Secondary sexual characteristics and deformities were observed under a dissecting scope. Growth and condition factors [ $\text{CF} = 100 \times (\text{weight g})/(\text{length cm})^3$ ], a measure of the body index, were determined by measurement of body weight and total length to the nearest 0.1 mg and 1.0 mm, respectively. Immediately after measurement, individual fish were longitudinally divided into two identical sections with a surgical blade and fixed in 10% neutral buffered formalin for histopathological analysis.

#### 2.3.2. Histopathology

Fixed samples were dehydrated in a graded ethanol series and embedded with both surgically cut sections face down in paraffin. Serial longitudinal sections (3–5  $\mu\text{m}$ ) were stained with hematoxylin and eosin, and lateral views of gonads, livers, and

thyroid glands were examined under a light microscope for lesions. Lesions were qualitatively described and semiquantitatively scored as not present, mild, moderate or severe.

#### 2.4. Data analyses

Growth data from normal controls and solvent controls were analyzed by *t*-tests assuming equal variance using STATISTICA 6.0. If no differences were found, these groups were pooled for subsequent analysis. When differences were found, the control group without the solvent was excluded from further analyses. If growth data were normally distributed, treatment and control groups were compared using one-way analysis of variance (ANOVA) and Fisher LSD pairwise multiple comparison test. If normality tests failed, we used Kruskal–Wallis one-way ANOVA followed by the Dunnett's multiple comparison test (USEPA, 2002). In the case of significant mortality, survival data were analyzed by the Spearman–Karber method to obtain 96 h-LC<sub>50</sub> estimates with a 95% confidence interval. We

used the  $P < 0.05$  level of significance. Results are expressed as 'mean  $\pm$  standard deviation'.

### 3. Results

#### 3.1. Water quality

The mean (range) water quality characteristics during both tests were pH, 7.72 (range 7.3–8.2); hardness, 120 (100–160) mg/l as CaCO<sub>3</sub>; alkalinity, 92.7 (80.0–100.0) as CaCO<sub>3</sub>; and dissolved oxygen, 7.24 (6.4–8.1) mg/l. Ammonia levels in the test solutions were negligible (<0.001 mg/l) during the exposures.

#### 3.2. Survival, growth, and gender effects

Mortality (64%) in 1-week-old male medaka larvae exposed to 3.2 mg/l of FLU was significant. The 96 h-LC<sub>50</sub> for males was 1.92 mg/l (95% CI 1.30–2.82). No significant mortality was observed in females exposed to FLU and females and males

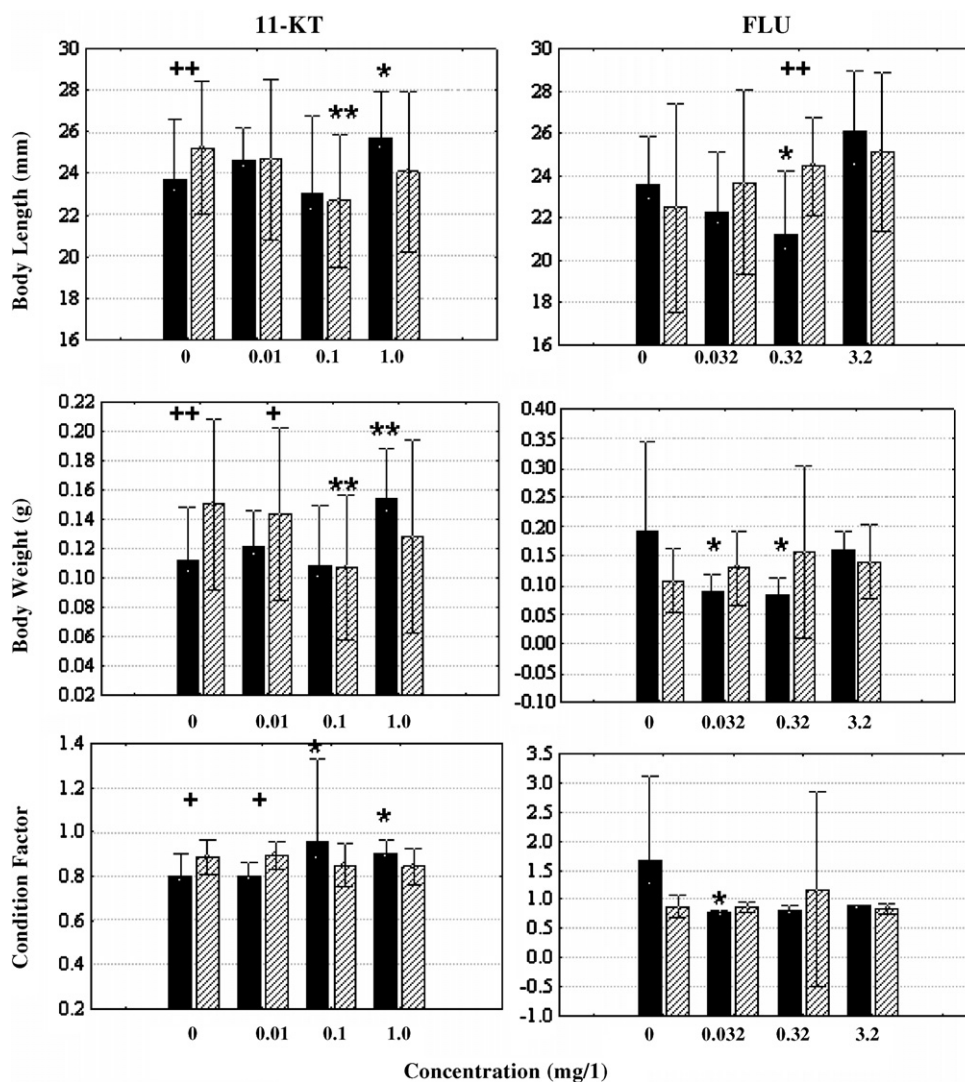


Fig. 1. Growth effects of 11-ketotestosterone (11-KT), a reference androgen, and flutamide (FLU), a reference antiandrogen, on Qurt medaka 3-month post-exposure. Solid rectangles and dashed rectangles represent male and female data, respectively. Statistically significant differences between solvent control group and treated fish of the same gender are denoted by \*\* $p < 0.01$  and \* $p < 0.05$ . Statistically significant differences between genders are denoted by ++ $p < 0.01$  and + $p < 0.05$ .

exposed to 11-KT after 96-h of exposure or at the end of a 90-day post-exposure period.

Treatment-related effects on growth in males and females exposed to 11-KT were significant (Fig. 1). Males and females treated with 0.1 mg/l of 11-KT showed growth effects. Females decreased significantly in body size ( $p < 0.01$ ) and body weight ( $p < 0.05$ ), while males had a higher condition factor compared to control groups (Fig. 1). Additional gender-specific growth effects were observed after exposure to the highest concentration of 11-KT (1.0 mg/l). Males in this treatment group were significantly larger ( $p < 0.05$ ) and heavier ( $p < 0.01$ ) than control males whereas females tended to have lower, but not significantly different, body weights than solvent controls with increasing 11-KT exposure. These gender-specific differences were also reflected in the condition factor. Males exposed to medium (0.1 mg/l) and high (1.0 mg/l) 11-KT levels had higher condition factors ( $p < 0.05$ ) than control males. On the other hand, females exposed to high (1.0 mg/l) 11-KT levels had lower condition factors ( $p < 0.05$ ) than control females.

Treatment-related growth effects in males exposed to FLU were significant (Fig. 1). Males exposed to 0.32 mg/l FLU were smaller than control males ( $p < 0.05$ ). Males treated with 0.032 and 0.32 mg/l FLU concentrations had lower body weights than control males ( $p < 0.05$ ). In addition, males treated with 0.032 mg/l FLU had a lower condition factor than control males ( $p < 0.05$ ). Females exposed to FLU had no statistically significant effects on growth.

### 3.2.1. Histopathology

Histological lesions were prevalent in the gonads and thyroid glands, but not in the livers, of both sexes 90-day post-96-h of exposure to 11-KT (Table 1; Fig. 2A–D) and FLU (Table 1; Fig. 3A–E). The most prevalent lesions observed in 11-KT treat-

ments were thyroid follicular cell hypertrophy (TFH) in males and females (Figs. 2A–C). TFH is characterized by increased size of follicular cells from a short cuboidal to a tall columnar shape and have large nuclei which reflects an increase in functional cell mass and metabolic activity. Males were twice more likely than females to show TFH after 11-KT treatment. Higher incidences of thyroid lesions were observed in males exposed to 0.1 mg/l of 11-KT than females. Females showed germ cell necrosis at lower 11-KT concentrations (0.01, 0.1 mg/l) than males (1.0 mg/l). Severe ovarian atresia was observed at low (0.01 mg/l) (Fig. 2D) and high (1.0 mg/l) 11-KT concentrations in females. The most prevalent lesions observed in FLU treatments were severe germ cell necrosis, testis-ova, and thyroid follicular cell hypertrophy in males (Fig. 3A–E). Flutamide induced testis-ova (0.32, 1.0 mg/l), ovarian cell necrosis (0.32 mg/l), and disrupted spermatogenesis (0.32, 3.2 mg/l) in males. Higher incidences of lesions, including TFH (Fig. 3B) and testis-ova (Fig. 3D), were observed in males exposed to 0.32 mg/l of FLU than in females. The lowest observed effective concentration (LOEC) for testis-ova induction in Qurt medaka males was 0.32 mg/l. Testis-ova was also observed in one male exposed to 1.0 mg/l of FLU. In addition, this male showed hyperplasia and hypertrophy of thyroid follicle. Severe germ cell necrosis, characterized by deformed spermatids (ST) and spermatozoa (SZ), occurred in a medaka exposed to 0.32 and 3.2 mg/l FLU (Fig. 3D and E).

## 4. Discussion

The presence of significant mortality (64%) in 1-week-old male medaka larvae exposed to 3.2 mg/l of FLU underlines the importance of gender-specific differences in survival. The LC<sub>50</sub>-96 h for FLU on Qurt medaka males (1.92 mg/l; 95% CI

Table 1  
Effect of 11-KT and FLU on adult gonad and thyroid histology of Qurt medaka after a 96-h exposure at the 1-week-old larval stage<sup>a</sup>

	0	0.01	0.1	1.0
11-KT (mg/l)				
Gonads				
Male				2 GCN (++)
Female		1 GCN (++); 1 OA (+++)	1 GCN (++)	1 OA (+++)
Thyroid gland				
Male		5 TFH (++)	3 TFH (++); 2 TFH (+++) <sup>a</sup>	2 TFH (++); 2 TFH (+++) <sup>b</sup>
Female		2 TFH (++)	1 TFH (++)	4 TFH (++)
	0	0.032	0.32	3.2
FLU (mg/l)				
Gonads				
Male			1 GCN (+++); 1 TO	1 GCN (+++)
Female				4 OA (++); 1 OA (+++)
Thyroid gland				
Male			1 TFH (++)	1 TFH (+++)
Female				

<sup>a</sup> The histological analysis was performed on gonads and thyroids of 15 animals/group/gender; GCN = germ cell necrosis; OA = oocyte atresia; TO = testis-ova; TFH = thyroid follicular cell hypertrophy. Semiquantitative scoring of histological lesions are denoted as follows: – = normal; + = mild (1–10 GCN; 1–2 OA); ++ = moderate (10–20 GCN; 3–5 OA); +++ = severe (>20 GCN; >5 OA); in addition to TFH, hyperplasia of follicular cells was observed in 11-KT-treated fish, 1 in the 0.1 mg/l and 2 in the 1.0 mg/l groups.

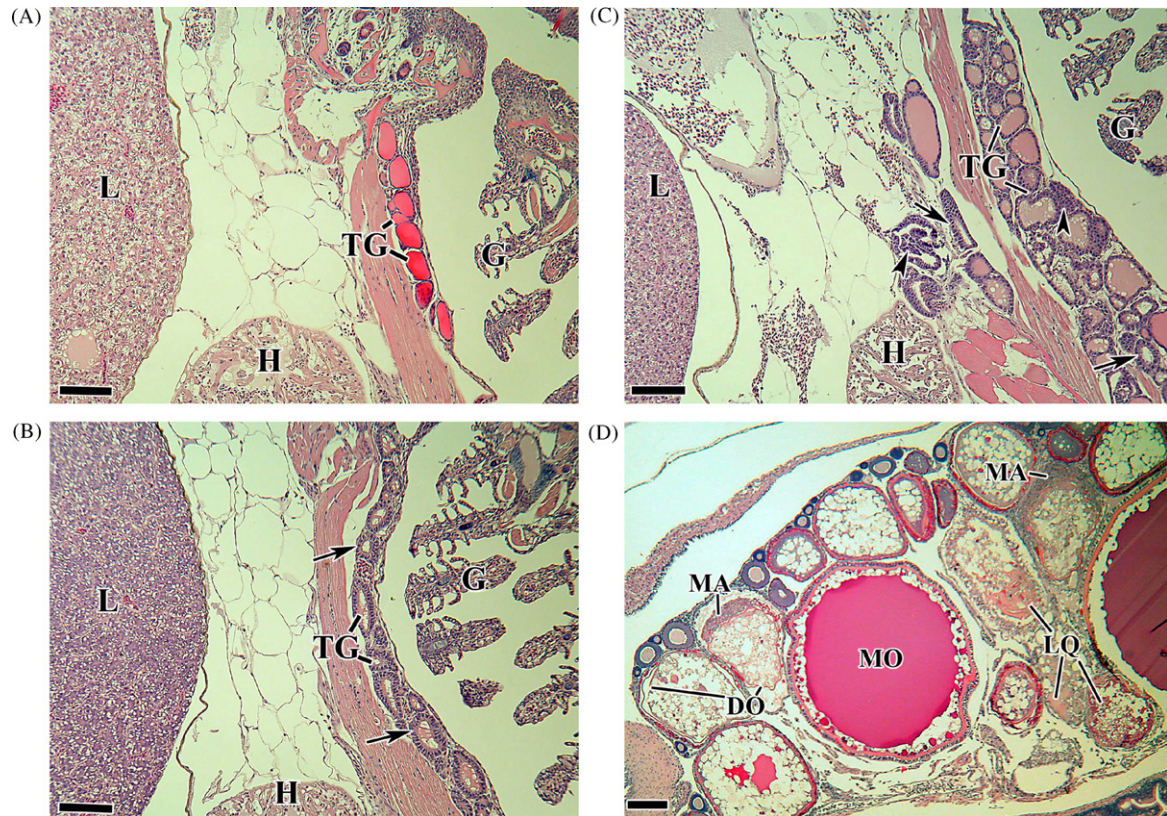


Fig. 2. Histopathological lesions of 3-month-old Qurt medaka previously exposed to 11-ketotestosterone (11-KT). (A) Normal thyroid gland morphology in a male medaka; (B) thyroid follicular cells hypertrophy (arrows) in female medaka exposed to 0.01 mg/l 11-KT, and (C) thyroid follicular cells hypertrophy (arrow) and cell hyperplasia (arrowheads) in male medaka exposed to 0.1 mg/l 11-KT (bar = 50  $\mu$ m). (D) Severe ovarian atresia indicated by the infiltration of macrophage aggregates (MA), liquefaction of oil and yolk granules (LQ), and degeneration of chorion and oocyte (DO) in medaka exposed to 0.01 mg/l 11-KT (bar = 200  $\mu$ m). TG = thyroid gland; L = liver; H = heart; G = gill; MO = mature oocytes.

1.30–2.82) is below the 3.6 mg/l estimated by Hagino et al. (2001) for newly hatched larvae. In addition, the latter value is above the highest FLU concentration used in the present study and more than three times greater than a biologically effective concentration for FLU (1.0 mg/l). Therefore, there is a large difference between the FLU concentration that causes significant mortality and that which causes endocrine disrupting effects in Qurt strain medaka. The absence of significant mortality after most 11-KT and FLU 96-h treatments suggest that acute toxicity testing is not particularly useful for endocrine disruption assessments. Acute exposures have been suggested as a first tier approach to identifying endocrine disrupting substances (Parrott et al., 2001).

In general, our results indicated that brief (96-h) exposures to 11-KT and FLU were capable of causing persistent effects on growth and reproduction of Qurt medaka. Growth and reproduction could be influenced by early life stage starvation during the 96-h test, but such effect was not evident in the control group. Sublethal effects on Qurt medaka we observed were consistent with biological effects attributed to endocrine disruption in wild freshwater fish (Jobling and Tyler, 2003). These effects include: altered growth and development, intersexuality, inhibited spermatogenesis, increased ovarian atresia, and thyroid alterations. Our findings suggest that the present combination of exposure

regime and sensitivity periods of Qurt medaka probably induce population-level effects.

Growth effects on Qurt medaka exposed to 11-KT and FLU were significant. Fish growth is an indicator of general health and could signal chronic responses to toxic exposure (Heath, 1987). In our study, a medium concentration of 11-KT (0.1 mg/l) caused a reduction in growth in females. A high concentration of 11-KT (1.0 mg/l) stimulated male growth as indicated by all growth parameters we measured. Gender-specific differences were more significant in FLU treatments. Overall, FLU-treated males had significantly impaired growth while female growth was not significantly affected by the same treatment. Our findings underscore the importance of evaluating gender dose-responses separately to characterize androgen disruption.

Differential metabolism of 11-KT and FLU by males and females could partially contribute to the gender-specific effects on growth. In vitro studies with fish microsomes indicated that medaka males metabolized testosterone at a rate 10-fold greater than that of females (Patyna et al., 1999). Testosterone hydroxylation yields potent metabolites that could lead to a higher degree of masculinization of exposed female fish. For example, in vivo studies based on dietary exposure to high concentrations of 11-KT stimulated a secondary sex character (37  $\mu$ g/g of food)

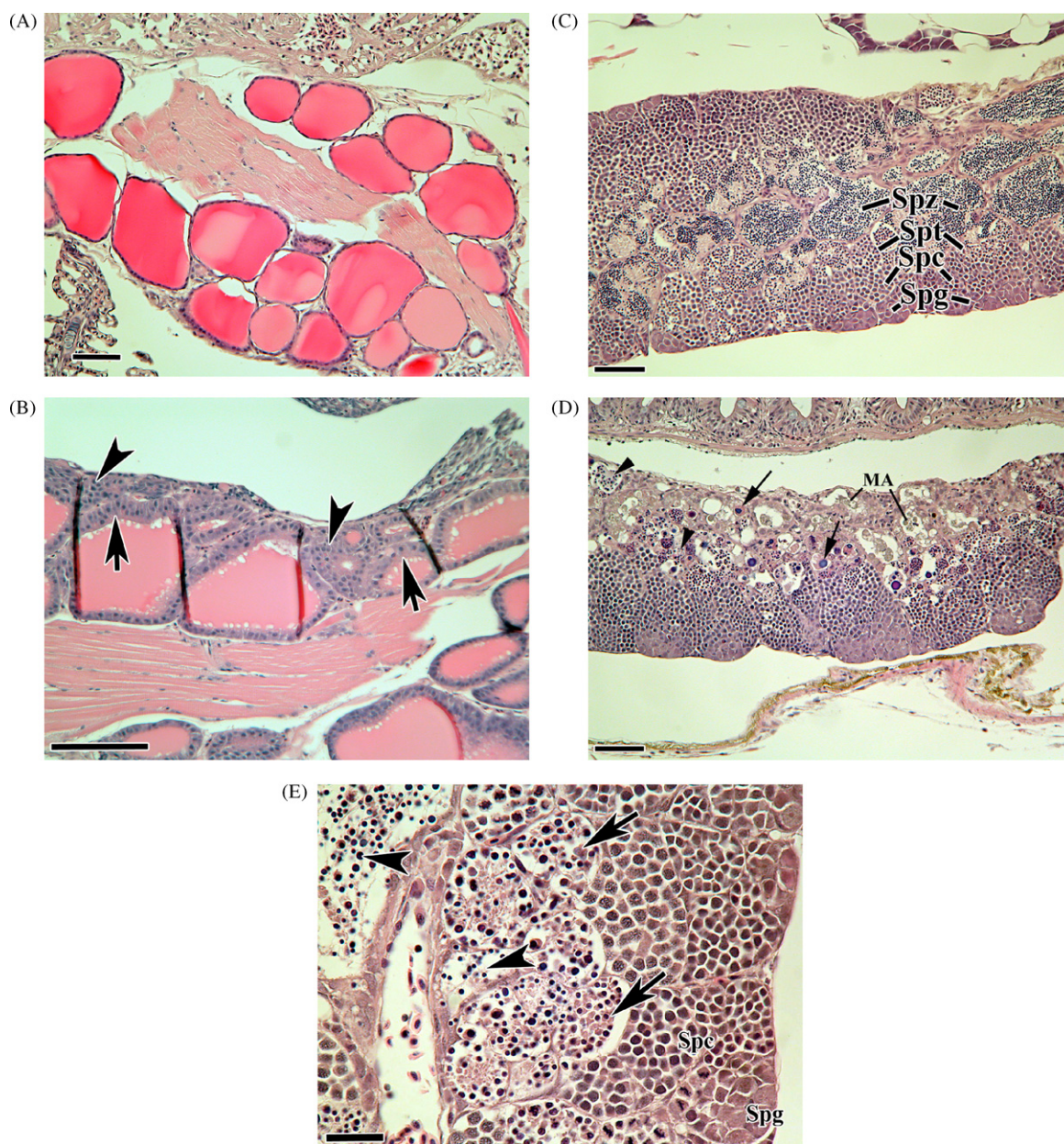


Fig. 3. Histopathological lesions of 3-month-old medaka previously exposed to FLU. (A) Normal thyroid gland in control male showing single-layered cuboidal follicular cells, bar = 50  $\mu$ m and (B) thyroid follicular cells hypertrophy (arrows) and hyperplasia (arrowheads) in male previously exposed to 0.32 mg/l FLU, bar = 50  $\mu$ m. (C) Normal testis of control male showing several reproductive stages in spermatogenesis (Spg = spermatogonia; Spc = spermatocytes; Spt = spermatids; Spz = spermatozoa) bar = 50  $\mu$ m, and (D) ovary in a male exposed to 0.32 mg/l FLU. Both severe male germ cell necrosis (arrowheads) and ova cell necrosis (arrows) were observed in this intersex fish (MA = macrophage aggregations) bar = 50  $\mu$ m; (E) severe germ cell necroses indicated by the presence of deformed and necrotic spermatids (arrows) and spermatozoa (arrowheads) were observed in a male exposed to 3.2 mg/l FLU. Compared to the normal testis shown in (A), the spermatozoa are sparse reflecting a low sperm count. Spg = spermatogonia; Spc = spermatocytes (bar = 20  $\mu$ m).

and reversed genetic females to phenotypic males (110  $\mu$ g/g food) in d-Rr strain medaka (Hishida and Kawamoto, 1970). On the other hand, mammalian studies with FLU indicate that 2-hydroxyflutamide is a much more potent antiandrogen than the parent FLU (Moguilewsky et al., 1986). Since fish possess the enzymatic systems necessary for this biotransformation (Jensen et al., 2004), it is likely gender-specific effects observed in Qurt medaka exposed to FLU are due to differential production of 2-hydroxyflutamide in both sexes.

Androgen disrupting effects will depend on the selected androgen. In our study, 11-KT was selected instead of methyltestosterone (MT) because it is a non-aromatizable androgen. Since 11-KT is not converted to estradiol by P450 aromatase, the biological effects observed in fish should be those expected from a pure androgenic agent. Exposure to MT induces the development of male secondary sex characteristics as well as formation of testis-ova in medaka (Seki et al., 2004). In another study, only medaka exposed as newly hatched fry or at 1-week-post-

hatch displayed intersex gonads following 0.1 mg/l testosterone (Koger et al., 2000). Formation of testis-ova in genetically male medaka could result from the conversion of part of MT to an estrogen by aromatase. Similarly, the partial feminization of sexually undifferentiated, juvenile fathead minnows exposed to waterborne MT (Zerulla et al., 2002) and the induction of testis-ova in MT-exposed zebrafish males (Örn et al., 2003) could be due to estrogen synthesis from MT aromatization.

Flutamide is a non-steroidal therapeutic agent for treatment of human prostate cancer (Kolvenbag et al., 2001) that can function as an antiandrogen in mammals (Bayley et al., 2002) and affects sexual differentiation and reproduction in fish. In fathead minnow, observed effects of FLU are consistent with an antiandrogenic mode of action due to competitive inhibition of the androgen receptor (Ankley et al., 2004; Jensen et al., 2004). In adult guppies, high concentrations of FLU exposure resulted in adverse effects to the testis structure (Kinnberg and Toft, 2003). In medaka, a sex-reversal test developed for the d-rR and S-rR strains has been applied to a variety of endocrine active chemicals, including FLU. Flutamide exhibited neither estrogenic nor androgenic actions on S-rR strain medaka at and below 1.0 mg/l (Hagino et al., 2001, 2002). However, antiandrogenic effects of FLU on medaka were suggested by the absence of female to male sex reversal after simultaneous exposure of larvae to FLU and effective concentrations of methyltestosterone (Hagino et al., 2002). Recent studies identified antiandrogenic effects of FLU on adult medaka (Kang et al., 2006). The lack of FLU effects in previous medaka sex-reversal assays (Hutchinson et al., 2003) could be due to the choice of strain, different timing of exposure, duration of exposure and post-exposure grow-out period prior to fish sampling. It is possible that medaka strains show differential sensitivity to FLU. We conducted 96-h instead of 21 days of exposure, 1-week-old larvae instead of newly hatched larvae, and 3-months instead of a 2-week period prior to fish sampling for growth and histopathological analysis. The observed 96 h-LOEC for testis-ova development in Qurt medaka males (0.32 mg/l) corresponded closely to the 21-day-LOEC (0.202 mg/l) reported by Kang et al. (2006). Our study confirmed some antiandrogenic properties of FLU on medaka males while providing new evidence of histological abnormalities in medaka germ cells.

Flutamide treatment induced mild germ cell necrosis in medaka males and ovarian atresia was observed in an intersex male and a female. Germ cell necrosis, severe inflammation at the spermatids stage, and absent or deformed spermatozoa were observed in a male treated with FLU (0.32 and 3.2 mg/l) (Fig. 3D and E). Germ cell necrosis alone was observed in one out of 15 fish exposed to a FLU concentration one order of magnitude lower (0.32 mg/l). In guppies, FLU reduced the number of spermatogenic cysts by blocking transformation of spermatogonia into spermatocytes in a dose-dependent fashion, but did not impede spermatocyte transformation into spermatozoa (Kinnberg and Toft, 2003). These results suggest that FLU could potentially impair fertility by disrupting male germ cell production in adult Qurt medaka. In contrast, medaka males exposed to FLU concentrations up to 0.787 mg/l for a longer term (21-d) had normal spermatogenesis (Kang et al., 2006). In our study,

ovarian atresia was identified in a female Qurt medaka exposed to the highest concentration (3.2 mg/l) of FLU. Ovarian atresia had only been reported in female fish in the wild (Jobling and Tyler, 2003). This is the first report of histological abnormalities in the ovaries of any female medaka exposed to FLU. On the other hand, the occurrence of ovarian atresia in an intersex male (Fig. 3D) suggests a mechanism by which intersexuality can be reversed in adult males.

Exposure to ADCs or other EDC classes can induce testis-ova or impair spermatogenesis, as shown in the present study. However, testis-ova induction will not necessarily impair reproduction of affected individuals (Mills and Chichester, 2005). For instance, medaka males that developed testis-ova after treatment with a range of waterborne estrogenic compounds were functionally male, still capable of producing mature spermatozoa and egg fertilization (Gray et al., 1999; Koger et al., 2000; Kang et al., 2002; Seki et al., 2004; Balch et al., 2004). Nevertheless, medaka males appear to be the most sensitive species for determining the capability of an EDC to induce intersex in laboratory models (Mills and Chichester, 2005). Other small fish models, such as zebrafish, may only exhibit transient formation of intersex gonads during a developmental window following hatch (Mills and Chichester, 2005).

Intersex medaka females were not observed after androgen exposure. Koger et al. (2000) suggest that female medaka masculinized by testosterone had sufficient differentiation of germ cells by day 21 to alleviate the effects of a 6-day exposure to androgens. On the other hand, when mature oocytes were incubated before fertilization with a nonaromatizable androgen, methylidihydrotestosterone (MDHT), it induced sex reversal of female to male (Iwamatsu et al., 2006). These results suggest that the critical window of exposure for permanent or organizational effects to occur in medaka females exposed to androgens might occur before fertilization. In our study, we exposed 7-day-old females to an androgen during a 4-day period and sampled at 90-day post-hatching. Hence, gonad disruption due to androgen exposure could have been reversed or alleviated as differentiation advanced during the post-exposure period and was not detected in sexually mature females. Alternatively, Qurt medaka females could be less sensitive to gonad disruption by androgens than thyroid disruption at this stage.

In the present study, thyroid follicle hyperplasia and hypertrophy in Qurt medaka are manifestations of thyroid disruption as a result of exposure to ADCs. In field studies, thyroid hyperplasia in salmon and herring gull populations was found in select regions of the Great Lakes (USA) (Leatherland, 1992). Thyroid hyperplasia in fish is a likely indicator of altered levels of the thyroid hormone, triiodothyronine (T3). The goiter condition may indicate that the fish is unable to produce T3. Triiodothyronine appears to be the most biologically active thyroid hormone which is under the control of the hypothalamus and pituitary (Sullivan et al., 1989; Arcand-Hoy and Benson, 1998). In fish, thyroid hormones have a major role on regulation of growth and development that can be affected as a result of xenobiotic exposure. For instance, estradiol may inhibit the formation of T3 from thyroxine (T4) in fish (Cyr and Eagles, 1996). Since, T3 is believed to play a role in testicular and oocyte growth

and maturation, low T3 levels can inhibit testicular growth. Few studies have addressed the effect of xenobiotic exposure on thyroid hormone levels (e.g. Ruby et al., 1993; Van der Ven et al., 2006). In the present study, the incidence of thyroid disruption was twice more likely in males than females in 11-KT and FLU treatments. It is likely that thyroid follicle hyperplasia and hypertrophy in Qurt medaka are compensatory responses to low T3 levels induced by ADC treatment in both genders, but the mechanism is currently unknown. We hypothesize that the administration of exogenous ADCs could affect endogenous T3 levels via feedback mechanisms on the release of hypothalamic and pituitary hormones.

## 5. Conclusion

Simultaneous occurrence of gonad and thyroid disruption in 11-KT- and FLU-exposed fish suggests the likelihood of cross-talk between hypothalamic-pituitary-gonadal and thyroidal axes in Qurt medaka. Therefore, our results underline the importance of fish early life stage tests for detecting the interaction of ADCs with thyroid hormone systems in both genders. Moreover, our approach to ADC testing with Qurt strain medaka might be optimal to screen wild fish species for persistent or unstable substances with a limited or episodic release that result in a short-term acute exposure.

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